Fishery Technology, 42(2):183-190, 2005

Fishery Technology
2005, Vol. 42(2) pp : 183 - 190

Bacterial Profile of Black Clam (*Villorita cyprinoides* var. *cochinensis*) and Clam Harvesting Waters from Vembanad Lake in Kerala (India)

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The bacterial flora of black clam (*Villorita cyprinoides* var. *cochinensis*) and water samples collected from three clam harvesting areas in Vembanad lake (Kerala, India) were studied. Samples were examined for total aerobic mesophilic count, psychrotrophic count, Vibrios and indicator bacteria. The mean mesophilic counts were in the range of 5.0-5.7 $\log_{10}$cfu ml^{-1} and 5.6-6.4 $\log_{10}$cfu g^{-1} respectively for water and clam samples. The shellfish collected from Vembanad lake showed faecal contamination at levels which did not conform to legal standards. The densities of enterococci and *Clostridium perfringens* were higher in clams than in the growing waters indicating bioconcentration of these organisms in clams. The bacterial flora on newly caught clam consisted of a variety of bacteria of which 28% were Gram-positive and 72% were Gram-negative. *Vibrio* and *Aeromonas* together formed 46% of the total mesophilic flora. *Vibrio* species isolated were *V. fluvialis, V. furnissi, V. metschnikovii*. Among *Aeromonas* species, *Aeromonas hydrophila, A. veronii* biovar. *sobria, A. media, A caviae* were isolated. The remaining Gram-negative genera in the flora belonged to *Acinetobacter, Shewanella, Moraxella* and *Pseudomonas*. The Gram-positive flora of clam was constituted by genera *Bacillus, Micrococcus, Corynebacterium* and *Arthrobacter*. High prevalence of *Escherichia coli*, faecal Streptococci *and C. perfringens* in water and clam indicates high degree of faecal pollution of the harvesting areas. The isolation of potentially pathogenic bacteria from clams indicates a risk for health of people consuming and also handling raw seafood.

Key words : **Bacterial flora, indicator bacteria, mesophilic count, black clam, *Villorita cyprinoides* var. *cochinensis*, clam harvesting waters, Kerala**

Commercial landings of the molluscan bivalve clams in India were estimated at 45412 tons in recent years, the bulk of the production from Kerala (73%) mostly by the Vembanad and Ashtamudi lakes. Black clam, *Villorita cyprinoides var cochinensis*, accounts for 64% of the production (Benzam, 1999). Clams represent an important food source in many parts of the world, particularly in Far East, South America and Europe and awareness in its safety value has been increasing. The export of frozen clam meat from the country in 2000 was 287 tons valued at Rs.1.3 crores. The other products being exported are dried clam meat and freeze-dried clam (MPEDA, 2001). Clam meat is widely used as feed in aquaculture.

Infectious disease outbreaks such as typhoid fever, cholera following consumption of raw or inadequately processed shellfish continue to occur world wide (Richards, 1987; 1988., West, 1989, Rippey, 1994). Bivalves being filter feeders, accumulate bacteria from the surrounding waters. Incidence of *Salmonella* (Frasier *et al.*, 1984., Varma *et al.*, 1988) and strains of *Vibrio cholerae* (Deopola *et al.*, 1983) in clam meat has been reported. Depuration of bivalves reduces the number of bacteria of faecal

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origin to insignificant levels (Balachandran & Surendran, 1984., Reilly & Barile, 1987). Types and levels of bacterial populations associated with clam are useful indicators of quality and safety of clam.

The objective of the present investigation was to determine the bacterial flora of black clam *Villorita cyprinoids var. cochinensis* harvested from Vembanad lake (Kerala) with a view to assessing the risks associated with the consumption of clams and also the risks associated with the use of clam meat as feed in shrimp aquaculture. The study is essential to develop safe handling practices for the production of clam safe for human consumption.

Materials and Methods

Live clams, *Villorita cyprinoids* var. *cochinensis* were collected from Vembanad lake, Kerala (on the southwest coast of India), mainly from three sites Perumpadappu, Udayamperoor and Vypeen, during the period July 2000, February 2001 and September 2001. Two clam samples were collected each time from each site and placed in sterile polythene bags. Water from the same area of harvesting of clams were separately collected in sterile glass bottles. All the samples were transported to the laboratory in an insulated ice-box for analyses within 2-4 h of collection. The clam samples were cleaned, aseptically shucked with a sterile knife. The meat and liquor were taken from 20 clams randomly selected from each sample lot and 25g sample was analysed. Water temperature, pH, dissolved oxygen and salinity were determined according to APHA (1998).

Bacteriological analysis

Clam sample (25 g) was homogenized for 1 min in a stomacher blender (Seward, U.K.) with 225 ml physiological saline for bacteriological analyses. Standard methods were followed for enumeration, isolation and identification of bacteria (FDA, 1998). Clam homogenates and water samples were serially diluted and plated on Tryptone Soya Agar (TSA, Oxoid, U.K.) for total aerobic mesophilic plate counts (TPC) at 37°C, 30°C (2-3 d) and 22°C (3-5 d). Psychrotrophs were determined on TSA at 7°C for 10 d. *Vibrio* and *Staphylococcus aureus* were enumerated according to the method of FDA (1998) and characteristic colonies were confirmed by biochemical tests (FDA, 1998; Alsina & Blanch, 1994).

Coliform, faecal coliforms, *Escherichia coli*, Enterococci and *Clostridium perfringens* numbers were determined by a 5 replicate tube MPN (Most Probable Number) procedure for water and by a 3 replicate tube MPN procedure for clam and confirmation of typical colonies (APHA, 1998; West, 1989).

A total of 176 mesophilic bacterial strains were randomly selected and isolated from water and clam samples. The bacterial strains picked from TSA plates incubated at 30°C were purified and tested for Gram reaction, cell morphology, catalase and oxidase reactions, motility, oxidation / fermentation test and presence of spores. They were then grouped according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984, Sneath, Mair, Sharpe & Holt, 1986), further tested for the most relevant characteristics of each group and identified using the above schemes and key schemes proposed by several authors for identification (Allen, Austin & Colwell, 1983, Austin, 1988, Kirov, 1997).

TPC were expressed as CFU g^{-1} for clam and as CFU ml^{-1} for water. TPC's were transformed to $\log_{10}$ values before statistical analysis. Analysis of variance was performed using the statistical tool package of Microsoft Excel 97 software. Student's t test analysis was used to evaluate the significance of differences between means of microbial counts performed in water and clam samples. $P < 0.05$ was considered statistically significant.

Results and Discussion

The three sites selected in this study represented the clam harvest areas in Vembanad lake in Kerala. Physico-chemical parameters varied among the three sites. Site 1 had higher salinity than sites 2 and 3 (Table1). Dissolved oxygen of surface water was slightly higher in site 2 than sites 1 and 3. Surface temperature in all the sites ranged from 28 to 32°C.

Table 1. Physico-chemical parameters of water from the three clam harvesting Sites in Vembanad lake in Kerala

Location	Temperature (°C)	pH	Dissolved Oxygen ml/l	Salinity (ppm)
Site 1	30±2°C	7.0-7.3	5.8-6.1	18.6-25
Site 2	29±1°C	6.9-7.1	6.0-6.3	10.3-18.2
Site 3	30±2°C	7.2-7.8	5.1-5.6	8.3-14.6

*Site 1 - Perumpadappu, 2 - Udayamperoor, 3 - Vypeen

Bacterial Levels

The counts of mesophilic bacteria, psychrotrophic bacteria and *Vibrio* on clams and in the waters collected from the three harvesting areas in Vembanad lake (Kerala, India) were determined and the results are presented in Table 2. The levels of mesophilic aerobic bacteria in water ranged from 5.0 to 5.7 $\log_{10}$ cfu ml^{-1} at 37°C. Among the three sites, water and clam samples collected from site 2 had the lowest TPC. Aerobic plate counts were high, indicating pollution of the harvest waters.

The mesophilic counts on clams were in the range of 5.6 to 6.45 $\log_{10}$cfu g^{-1} at 37, 30 and 22° C. Aerobic mesophilic counts at 37, 30 and 22°C for water and clam did not differ significantly ($P > 0.05$). These counts generally remained above the limit stipulated by FDA. Similar observations were made earlier (Vijayan *et al.* 1982., Surendran & Balachandran, 1984).

Fresh shellfish should have an APC less than 5×10^5 cfu g^{-1} (Clem, 1982). Clams taken from waters with high TPC contained increased TPC levels as reported earlier (Chai *et al.*, 1994). Cann (1977) reported TPC of clams (*Mercenaria mercenaria*) in the range of 10^3 to $10^8/cm^3$ at 20 and 37°C. and in 42 - 51% of the samples, the count exceeded $10^6/cm^3$ of tissue macerate.

The psychrotrophic bacterial counts on clams were significantly lower than the mesophilic counts ($P < 0.05$) indicating that a small fraction (<1%) of the microflora is to be characterized as psychrotrophic organisms.

Table 2. Mean Bacterial count of Clam (*Villorita cyprinoides*) and waters from the three harvesting sites in Vembanad lake (Kerala)

Bacteriological parameters	Mean Bacterial count: Water ($\log_{10}$ cfu ml^{-1}) Site1*	Site 2	Site 3	Clam ($\log_{10}$ cfug^{-1}) Site 1	Site 2	Site 3
Total Plate count-37°C	5.64±0.11**	5.02±0.08	5.49±0.64	6.37± 0.27	5.66±0.18	6.44±0.15
30°C	5.59±0.23	5.12±0.08	5.55±0.43	6.27±0.94	5.71±0.09	6.45±0.06
22°C	5.72±0.11	4.97±0.03	5.49±0.46	6.17±0.15	5.66± 0.02	6.40±0.09
7°C	3.07±0.02	3.00±0.02	3.11±0.07	3.95±0.16	2.97±0.11	2.53±0.21
Total Vibrios	3.47±0.11	2.79±0.08	3.71±0.15	5.14±0.24	4.23±0.03	4.47±0.15
sucrose positive	3.37±0.16	2.56±0.08	3.48±0.16	5.07±0.28	3.17±0.13	4.23±0.19
sucrose negative	2.73±0.13	2.4±0.08	3.31±0.14	4.29±0.29	4.19±0.04	4.10±0.10

*Site 1 - Perumpadappu., 2 - Udayam peroor, 3 - Vypeen ** standard deviation

Table 3. Mean counts (log_{10} MPN) of indicator bacteria in clam(*Villorita cyprinoides*) and water from the three harvesting sites in Vembanad lake (Kerala)

Bacteriological parameters	Mean Bacterial count: Water (log MPN 100 ml^{-1})			Clam (log MPN g^{-1})		
	Site1*	Site 2	Site 3	Site 1	Site 2	Site 3
Total coliforms	4.01±0.03**	4.05±0.9	3.75±0.21	3.99±0.04	4.09±0.05	3.89±0.15
Faecal coliforms	3.80 ±0.15	3.35±0.31	3.11± 0.07	3.42±0.24	3.11±0.07	2.57±0.09
Escherichia coli	3.35 ±0.31	3.41±0.24	2.85±0.19	2.99±0.04	2.98±0.32	2.17±0.13
Faecal streptococci	2.7±0.04	2.35± 0.31	2.48±0.17	2.72±0.06	2.57±0.09	2.85±0.19
Clostridium perfringens	1.42 ±0.24	1.01±0.17	0.80±0.5	1.59±0.45	1.11±0.07	0.98±0.68

* Site 1 - Perumpadappu, 2 - Udayamperoor, 3 - Vypeen ** standard deviation

The counts of *Vibrio* on clams collected from site 1 was higher (5.14 log $_{10}$ cfu g^{-1}) than that from sites 2 and 3 ($P > 0.05$). The densities of Vibrio was higher i.e., more than one log_{10} cfu, in clams compared to water ($P < 0.05$). *Vibrio* species on clams from site 1 were identified as *V. furnissi* and *V. mimicus*. In addition, clam from site 3, harboured *V. metschnikovi*. Clam from site 2 harboured *V. alginolyticus, V. fluvialis, V. mimicus, V. campbelli* and *V. anguillarum*. Among these, except *V. anguillarum* and *V.campbelli,* all others are associated with food-borne disease (FDA, 1998). *V. anguillarum* is a fish/shrimp pathogen (Inglis *et al.*, 1993). These organisms if present in high numbers in clams are of concern because they can be transmitted by the ingestion of raw or inadequately cooked seafood. These pathogenic species also enter shrimp/ prawn farms and farmed prawn through clam feed. Vibrio counts were high (>10^4 cfu/g) in clams (*Mercenaria mercinaria*) during summer months (Brenton *et al.*, 2001).

Indicator organism levels in growing water and in clam

The mean counts of indicator bacteria in clam and waters from the three harvesting sites in Vembanad lake (Kerala) are presented in Table 3. The faecal coliform and *E. coli* levels in water were above the normal limits (14 100ml^{-1}) set by EC (Anon, 1991) for unrestricted shellfish harvest. Surendran *et al.* (2002) reported comparatively higher values for *E. coli* in cochin backwaters. The total coliform, faecal coliform and *E. coli* counts on clams exceeded the limits (230 100g^{-1}) set by FDA (1997) and EC (Anon 1991) for shellfish sold for consumption.

The densities of enterococci and *C. perfringens* in the present study were higher in clams than in the growing waters indicating bioconcentration of these organisms in clams. *C. perfringens* counts <10^4 MPN 100g^{-1} were reported for unpurified bivalve shellfish (Madden *et al.*, 1986., Easterbrook & West, 1987). There are no

Table 4. Indicator organism density in clam and shellfish growing waters from Vembanad lake in Kerala

Location	Ratio of indicator organisms in clam to water***: Total coliforms	Faecal coliforms	*Escherichia coli*	Faecal Streptococci	*Clostridium perfringens*
Site1*	97	44	35	105	206
Site2	108	46	41	134	118
Site3	132	29	20	238	213

* Site 1 - Perumpadappu, 2 - Udayamperoor, 3 - Vypeen
*** ratio of indicator organisms in 100 g clam to 100ml water

established standards for using *C. perfringens* densities to assess the sanitary quality of shellfish or shellfish growing waters. *S. aureus* was detected in none of the water and clam samples analysed. Numbers of indicator organisms in shellfish-growing water reflect the quality or level of pollution in the shellfish.

Burkhardt and Calci (2000) showed an accumulation factor (mean ratio of the organism in shellfish compared to water) of 4.4 for faecal coliforms in oysters. Chai et al.(1994) reported bioconcentration of total coliforms by clams at 80 to 115 fold, faecal coliforms at 10 to 18 fold and *E. coli* at 7 to 12 fold. Accumulation factors can be as high as 25 to 30 for faecal bacteria (Reilly and Barile 1987). In the present study, bioaccumulation factor for total coliforms ranged from 97 to 132 and that for *E. coli* ranged from 20-41 for clam samples with out depuration (Table 4). Clam from site 3 had the lowest accumulation factor for faecal coliforms and *E. coli* and the highest factor for faecal streptococci and *C. perfringens*. Bioconcentration of *C. perfringens* by clams was at 118 to 213 fold. Burkhardt and Calci (2000) showed an accumulation of 58 to 245 for *C. perfringens* in oysters. Several investigators have reported significant reduction (99.7%) in *E. coli* levels during depuration (Reilly & Barile, 1987., Surendran & Balachandran, 1988). Therefore it is suggested that clams harvested from polluted waters should be depurated to reduce the numbers of faecal bacteria to acceptable level (230 $100g^{-1}$) before processing.

Bacterial flora

A total of 55 bacterial strains were identified from water (Table 5). The gram-negative flora formed 71% of the total mesophilic flora and dominated by genera *Vibrio*, *Aeromonas* and *Pseudomonas*. The bacterial flora of clam consisted of a variety of bacteria, of which 28% were Gram-positive and 72% were Gram-negative. The proportion of Gram-negative genera in the commensal flora varies considerably in clams harvested from the three sites (Table6). The bacterial flora of clam from site 2 and 3 was dominated by *Vibrio* (30%) where as that from site 1 was dominated by *Aeromonas* (33%). *Vibrio* species isolated were *V. fluvialis*, *V. furnissi*, *V. metschnikovii*. Among *Aeromonas*

Table 6. Composition of the bacterial flora of Black Clam *Villorita cyprinoids* from Vembanad lake in Kerala

Bacterial Genera	Distribution of the main bacterial genera		
	Site 1	Site 2	Site 3
Vibrio	6	11	10
Aeromonas	15	7	6
Enterobacteriaceae	-	3	1
Chromobacterium	1	1	-
Pseudomonas	3	1	2
Shewanella	4	3	2
Moraxella	1	-	2
Acinetobacter	3	2	3
Bacillus	5	4	5
Micrococcus	4	4	2
Arthrobacter	3	2	2
Staphylococcus	-	2	1
Total	45	40	36

*Site 1 - Perumpadappu, 2 - Udayamperoor, 3 - Vypeen

Table 5. Composition of the bacterial flora associated with water from the three clam harvesting sites in Vembanad lake in Kerala

Bacterial Genera	Distribution of the main bacterial genera		
	Site 1	Site 2	Site 3
Vibrio	4	4	6
Aeromonas	3	2	4
Enterobacteriaceae	-	1	2
Chromobacterium	1	1	-
Pseudomonas	1	2	1
Flavobacterium	2	1	-
Moraxella	1	-	2
Acinetobacter	-	1	2
Micrococcus	2	1	3
Bacillus	2	3	4
Total	16	16	24

*Site 1 - Perumpadappu, 2 - Udayamperoor, 3 - Vypeen species isolated, *Aeromonas hydrophila, A. veronii* biovar. *sobria, A. media, A caviae* were encountered. The remaining Gram-negative genera in the flora belonged to *Acinetobacter, Shewanella, Moraxella* and *Pseudomonas*. The Gram-positive flora of clam was constituted by genera *Bacillus, Micrococcus, Corynebacterium* and *Arthrobacter*. In addition, genus *Staphylococcus* was represented in the microflora of clam from site 2 and 3. Bivalves in their natural environment carry a commensal bacterial load, the composition of which may be mainly influenced by the quality and temperature of the waters in which they exist (West, 1989).

In general, the major genera of bacteria comprising the flora of clam was *Vibrio* and *Aeromonas* which together formed 46% of the total mesophilic flora. Their growth to undesirably high levels can be very rapid during post-harvest handling at ambient temperatures which presents a risk to public health as reported earlier (Colwell, 1984., West 1989., Tamplin, 1994., Brenton *et al.*, 2001). They are also recognized as the cause of wound and blood infections following lacerations to the skin acquired during handling of shellfish(Flynn & Knepp, 1987). Clam from site 3 had the lowest accumulation factor for faecal coliforms and *E. coli*. However, bioaccumulation of *Vibrio*, *C. perfringens* and faecal streptococci was higher in clams from site 3 compared to that from site 1 and 2.

Conclusions

The results demonstrate that the bacterial levels in clams harvested from the three sites in Vembanad lake and that harvested from other tropical and temperate waters are similar. Among the three sites examined, water and clam collected from site 2 showed lowest counts of mesophilic bacteria, *Vibrio*, Enterococci and *C. perfringens*. The high incidence of pathogenic bacteria should be regarded as a potential health concern when there is possibility of cross-contamination and also multiplication of these mesophilic bacteria during post-harvest handling at ambient temperatures.

Clam meat is widely used as cheaper feed in shrimp farms and it may contaminate farmed shrimp. It is recommended that clam meat, when used as feed, should be cooked to destroy the natural bacterial flora. Good handling practices should be followed immediately after harvesting clams to avoid public health risk. The results of the study indicates the need for monitoring shellfish that will be consumed raw or undercooked, not only for faecal indicators, but also for many species of *Vibrio* and *Aeromonas* and their virulence factors.

The authors are grateful to Director, CIFT for providing all facilities and for permission to publish this paper.

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